Single-nucleotide polymorphism haplotypes in the both proximal promoter and exon 3 of the APM1 gene modulate adipocyte-secreted adiponectin hormone levels and contribute to the genetic risk for type 2 diabetes in French Caucasians

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Adiponectin (ACRP30), an adipocyte-secreted protein encoded by the APM1 gene, is known to modulate insulin sensitivity and glucose homeostasis, those effects protecting obese mice from diabetes. Plasma adiponectin levels correlate well with insulin sensitivity in humans, and are decreased in both type 2 diabetes (T2D) and obesity. We screened for single-nucleotide polymorphisms (SNPs) the APM1 gene coding and 5’ sequences in 40 French Caucasians: 12 SNPs and 4 rare non-synonymous mutations of exon 3 were detected. The 10 most frequent SNPs were genotyped in 1373 T2D and obese French Caucasian subjects and in all subjects available from 148 T2D multiplex families. The screening for rare mutations of exon 3 was extended to 1246 T2D and obese French subjects and to the members of the 148 T2D multiplex families. A haplotype including SNPs −11391 and −11377, both located in the 5’ sequences, was associated with adiponectin levels ($P < 0.0001$) and with T2D ($P = 0.004$). The presence of at least one non-synonymous mutation in exon 3 showed evidence of association with adiponectin levels ($P = 0.0009$) and with T2D ($P = 0.005$). We failed to detect an association with insulin resistance indexes. Although family-based association analysis with T2D did not reach significance, our results suggest that an at-risk haplotype of common variants located in the promoter and rare mutations in exon 3 contribute to the variation of the adipocyte-secreted adiponectin hormone level, and may be part of the genetic determinants for T2D in the French Caucasian population.

INTRODUCTION

The worldwide epidemic of obesity is considered a major health problem, since excess and/or ectopic body fat deposition is a potent risk factor for insulin resistance, dyslipidemia and type 2 diabetes (T2D), leading to premature cardiovascular morbidity and death (1). There is now a large body of evidence that the adipose tissue is not only the major tissue for energy storage but is also an active endocrine organ, secreting a variety of proteins that notably influence the metabolism of the body and affect energy and glucose homeostasis (2). Among these proteins, tumor necrosis factor $\alpha$ (TNF-$\alpha$), plasminogen
activator inhibitor type 1 and possibly resistin (3–5), which are overexpressed in obesity states, are thought to contribute to the development of insulin resistance, a major risk factor for T2D and macrovascular complications. In contrast to these proteins, administration of leptin, the key component for regulation of food intake at the central level, prevents the occurrence of diabetes in a model of severely insulin-resistant lipodystrophic mice with overexpression of a truncated version of sterol-regulatory-element-binding protein 1c (6). Furthermore, leptin concentrations are elevated in obese human subjects; thus it is unlikely that leptin deficiency contributes to the development of diabetes in obese humans.

Little is known, so far, about putative adipocyte-specific secretory proteins that might protect the majority of obese and leptin-resistant subjects from the early development of T2D. Among the newly identified fat-secreted hormones suggested to modulate insulin sensitivity (5), the 30 kDa adipocyte complement-related protein (ACRP30/adiponectin), encoded by the APM1 gene, is only expressed in differentiated adipocytes (7). In rodents consuming a high-fat/sucrose diet, administration of a low dose of the globular head domain of ACRP30/adiponectin increased free fatty acid (FFA) muscle oxidation and caused weight reduction (8). Decreased adiponectin expression correlates with insulin resistance in genetic models of obesity and lipodystrophy, and these mice are rescued from severe insulin resistance by recombinant ACRP30 (9). ACRP30 administration abolishes hyperglycemia in diabetic mice and suppresses glucose production from adipocytes (10). In humans, ACRP30/adiponectin is one of the most abundant plasma proteins. Hypoadiponectinemia is reported in obesity and T2D, and ACRP30/adiponectin levels correlate with insulin resistance in different ethnic groups (11).

The human APM1 gene maps to the 3q27 region, where various quantitative trait loci (QTL) for the metabolic syndrome and a locus for T2D were reported (12–14), and is composed of three exons spanning 16 kb (15,16). We recently identified in the Japanese population 10 relatively frequent single-nucleotide polymorphisms (SNPs), two of them being associated with an increased risk of T2D (17). Here we report results of the screening of the APM1 gene in a Caucasian population. Serum adiponectin levels were determined, and association studies between several APM1 SNPs and adiponectin levels and T2D were conducted to evaluate the contribution of APM1 genetic variants to diabetes.

RESULTS

The 10 SNPs previously reported by Hara et al. (17) in Japanese subjects were detected in our study, together with additional two rarer SNPs: −11156 insCA 0.94/0.06 and −11043 C > T 0.98/0.02 (Fig. 1). We also found four rare non-synonymous mutations in exon 3: G84R, G90S, R92X and Y111H. Therefore, we extended the exon 3 screening to 1246 subjects and 148 T2D multiplex families. The frequencies of the G90S and Y111H variants were 0.9948/0.0052 and 0.9844/0.0156, respectively, and the remaining G84R and R92X were detected each in a single family. The R112C, I164T, R221S and H241P mutations reported in Japanese populations (15,17) were not detected in the current study. The R92X was only present in a 71-year-old non-diabetic spouse, and her adiponectin level was not available. The G84R mutation was present in two out of three diabetic subjects from the same sibship. Interestingly, the adiponectin levels adjusted for age, sex and body mass index (BMI) were respectively 2 and 2.6 times lower in subjects with the G84R mutation as compared with their wild-type sister. If all three sibs were diagnosed with diabetes after 50 years of age, only the one with the lowest adiponectin level was non-obese (BMI = 23 kg/m²).

A high heritability for plasma adiponectin levels was detected in our sample of 148 families (70%, P < 0.0001). As we hypothesized an effect of SNPs on the variation of adiponectin levels and on the risk of T2D, we genotyped the 10 common SNPs in our population. Genotype frequencies differed widely from those reported in the Japanese population (Table 1). SNPs +45 T > G (G15G) and +349 A > G were in complete linkage disequilibrium (D = 1); likewise for SNPs +712 G > A and +2019 delA (D = 0.99) (Table 2). Therefore, in both cases, only SNPs +45 and +2019 were investigated further. It is noticeable that the rare mutation G90S is closely associated with almost all the SNPs studied, except for SNPs −11426 and −11377. Conversely, the other rare mutation Y111H was only associated with SNPs −11426, +45 and +349. We constructed haplotypes with the eight remaining frequent SNPs. Only 14 haplotypes had a prevalence >1%, and represented 90.5% of our population (Table 3).

Serum adiponectin levels were measured in a randomly chosen subset of 922 subjects. We found that adiponectin levels were correlated positively with age and negatively with BMI, and were higher in females (data not shown); therefore, in further analyses, adiponectin values were corrected accordingly. Higher adiponectin levels were associated with variant alleles of SNPs −11391 G > A (P < 0.0001), +45 T > G (P = 0.01), +276 G > T (P = 0.01) and +2019 delA (P < 0.0001), whereas variant alleles at SNP −11377 C > G (P = 0.0003) and G90S (P = 0.016) were associated with a lower adiponectin level. The Y111H variant presented a trend of association with a lower adiponectin level (P = 0.08).

In order to assess what are the functional SNPs, we successively analyzed the effects of the SNPs that were weakly associated with adiponectin levels (+45 and +276) in the subgroups of subjects that were wild-type for SNP −11391, SNP −11377 or SNP +2019. No association was then detected for either SNP +45 or +276 in all subgroups of subjects (P > 0.3). Indeed, haplotype analysis revealed that the putative effects of SNP +45 and +276 mostly resulted from a linkage disequilibrium with SNP −11391 or SNP −11377 (Tables 2 and 3). Owing to the low frequencies of G84R, G90S and Y111H, such analysis could not be individually conducted for these mutations. Therefore G84R, G90S and Y111H were pooled for analyses and recoded as ‘presence of at least one’ versus ‘absence of’ mutation in exon 3. The presence of at least one mutation in exon 3 was associated with a lower adiponectin level (P = 0.0009).

In subgroups wild-type for either SNP −11391 or SNP −11377, the presence of at least one mutation in exon 3 remained associated with a lower adiponectin level (P = 0.008 and 0.004 respectively). This was not true in the subgroup that were wild-type for SNP +2019,
reflecting a linkage disequilibrium between SNP +2019 and G90S ( \( D' = -1 \)) or Y111H ( \( D' = -0.68 \)).

We then constructed haplotypes including SNPs −11391, −11377 and +2019 in three subsets of subjects according to the tertiles of the adiponectin levels. Estimated frequencies of the risk haplotype G-G-insA (1–2–1) were 0.256, 0.220 and 0.181, respectively, in the low (1st), medium (2nd) and high (3rd) adiponectin level tertiles. Haplotype frequencies differed between the 1st and 3rd tertiles ( \( P < 0.0001 \)). Haplotype analyses including SNPs −11391 and −11377 and the rare mutations were inconclusive ( \( P = 0.2 \)) because of the low frequency of these mutations. Quantitative transmission disequilibrium testing (TDT) analysis in our 148 nuclear families showed transmission disequilibrium between

![Figure 1. Position of SNPs in the human APM1 gene.](image)

### Table 1. Distributions of genotypes and alleles for the 10 common SNPs, in normoglycemic (NDM) and type 2 diabetic (T2D) subjects

<table>
<thead>
<tr>
<th>Polymorphismsa</th>
<th>Genotypesb</th>
<th>Pb</th>
<th>Allelesb</th>
<th>Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>−11426 (−11414)</td>
<td>AA AG GG</td>
<td>0.59</td>
<td>A G</td>
<td>0.59</td>
</tr>
<tr>
<td>T2D 516 (83.7)</td>
<td>97 (15.8)</td>
<td>3 (0.5)</td>
<td>1129 (91.6)</td>
<td>103 (8.4)</td>
</tr>
<tr>
<td>NDM 473 (85.4)</td>
<td>77 (13.9)</td>
<td>4 (0.7)</td>
<td>1023 (92.3)</td>
<td>85 (7.7)</td>
</tr>
<tr>
<td>T2D 500 (80.6)</td>
<td>111 (17.9)</td>
<td>9 (1.5)</td>
<td>1111 (89.6)</td>
<td>129 (10.4)</td>
</tr>
<tr>
<td>NDM 579 (83.9)</td>
<td>106 (15.4)</td>
<td>5 (0.7)</td>
<td>1264 (91.6)</td>
<td>116 (8.4)</td>
</tr>
<tr>
<td>−11377 (−11365)</td>
<td>CC CG GG</td>
<td>0.09</td>
<td>A G</td>
<td>0.09</td>
</tr>
<tr>
<td>T2D 300 (48.4)</td>
<td>274 (44.2)</td>
<td>46 (7.4)</td>
<td>874 (70.5)</td>
<td>366 (29.5)</td>
</tr>
<tr>
<td>NDM 382 (55.3)</td>
<td>264 (38.2)</td>
<td>45 (6.5)</td>
<td>1028 (74.4)</td>
<td>354 (25.6)</td>
</tr>
<tr>
<td>−4041 (−4034)</td>
<td>AA AC CC</td>
<td>0.06</td>
<td>A C</td>
<td>0.06</td>
</tr>
<tr>
<td>T2D 248 (40.4)</td>
<td>288 (46.9)</td>
<td>78 (12.7)</td>
<td>784 (63.8)</td>
<td>444 (36.2)</td>
</tr>
<tr>
<td>NDM 295 (44.0)</td>
<td>315 (46.9)</td>
<td>61 (9.1)</td>
<td>905 (76.4)</td>
<td>437 (32.6)</td>
</tr>
<tr>
<td>−3971 (−3964)</td>
<td>AA AG GG</td>
<td>0.20</td>
<td>A G</td>
<td>0.20</td>
</tr>
<tr>
<td>T2D 403 (66.9)</td>
<td>186 (30.9)</td>
<td>13 (2.2)</td>
<td>992 (82.4)</td>
<td>212 (17.6)</td>
</tr>
<tr>
<td>NDM 475 (70.5)</td>
<td>187 (27.7)</td>
<td>12 (1.8)</td>
<td>1137 (84.3)</td>
<td>211 (15.7)</td>
</tr>
<tr>
<td>+45 (+45)</td>
<td>TT TG GG</td>
<td>0.90</td>
<td>T G</td>
<td>0.90</td>
</tr>
<tr>
<td>T2D 426 (73.2)</td>
<td>140 (21.4)</td>
<td>16 (2.7)</td>
<td>992 (85.2)</td>
<td>172 (14.8)</td>
</tr>
<tr>
<td>NDM 500 (73.9)</td>
<td>156 (22.9)</td>
<td>24 (3.5)</td>
<td>1156 (85.0)</td>
<td>204 (15.0)</td>
</tr>
<tr>
<td>+276 (+276)</td>
<td>GG GT TT</td>
<td>0.77</td>
<td>G T</td>
<td>0.77</td>
</tr>
<tr>
<td>T2D 323 (56.0)</td>
<td>203 (35.2)</td>
<td>51 (8.8)</td>
<td>849 (73.6)</td>
<td>305 (26.4)</td>
</tr>
<tr>
<td>NDM 387 (56.8)</td>
<td>220 (32.3)</td>
<td>74 (10.9)</td>
<td>994 (73.0)</td>
<td>368 (27.0)</td>
</tr>
<tr>
<td>+349 (+349)</td>
<td>AA AG GG</td>
<td>0.68</td>
<td>A G</td>
<td>0.68</td>
</tr>
<tr>
<td>T2D 426 (73.7)</td>
<td>136 (23.5)</td>
<td>16 (2.8)</td>
<td>988 (85.5)</td>
<td>168 (14.5)</td>
</tr>
<tr>
<td>NDM 510 (75.3)</td>
<td>146 (21.6)</td>
<td>21 (3.1)</td>
<td>1166 (86.1)</td>
<td>188 (13.9)</td>
</tr>
<tr>
<td>+712 (+712)</td>
<td>GG GA AA</td>
<td>0.17</td>
<td>G A</td>
<td>0.17</td>
</tr>
<tr>
<td>T2D 215 (33.3)</td>
<td>301 (46.7)</td>
<td>129 (20.0)</td>
<td>731 (56.7)</td>
<td>559 (43.3)</td>
</tr>
<tr>
<td>NDM 208 (37.7)</td>
<td>241 (43.7)</td>
<td>103 (18.7)</td>
<td>657 (59.5)</td>
<td>447 (40.5)</td>
</tr>
<tr>
<td>+2019 (+2019)</td>
<td>NN N delA delA delA</td>
<td>0.36</td>
<td>N delA</td>
<td>0.36</td>
</tr>
<tr>
<td>T2D 221 (33.7)</td>
<td>306 (46.7)</td>
<td>128 (19.5)</td>
<td>748 (57.1)</td>
<td>562 (42.9)</td>
</tr>
<tr>
<td>NDM 260 (36.2)</td>
<td>326 (45.4)</td>
<td>132 (19.5)</td>
<td>846 (58.9)</td>
<td>590 (41.1)</td>
</tr>
</tbody>
</table>

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aThe numbering reported by Hara et al. (17) based on a previous release of the Human Genome Working Draft, is given in parentheses.
bNumbers (frequencies, in %, are shown in parentheses).
the risk haplotype G-G (1–2) inferred for each individual and a lower adiponectin level (additive effect of −0.067, P = 0.0013).

Sib-TDT analyses with the rare mutations were not informative enough to report a valid P-value (maximum of four families with both alleles simultaneously).

In order to assess the putative contribution of APMI to the genetic risk for T2D, we looked for an association of diabetes with APMI SNPs and exon 3 rare mutations. Following a test with 1000 permutations, SNPs −11391, −11377, G90S and Y111H were individually associated with diabetes [P = 0.030, odds ratio (OR) = 0.79 (confidence interval 95%, CI = 0.606–1.06), P = 0.027, OR = 1.31 (CI = 1.06–1.63), P = 0.065, OR = 2.69 (CI = 1.14–6.36) and P = 0.006, OR = 2.48 (CI = 1.39–4.41), respectively], whereas SNP +2019 was not (P = 0.47). The presence of at least one rare mutation in exon 3 was also associated with T2D [P = 0.005, OR = 2.40 (CI = 1.45–3.95)]. Haplotypes including SNPs −11391 and −11377 from our whole population were constructed. Estimated frequencies of the risk haplotype G-G were 0.293 and 0.253 in the T2D and the normoglycemic groups, respectively, and differed between these two groups (P = 0.004). A similar association with T2D (P = 0.003) was observed when considering haplotypes including SNPs −11391, −11377 and the recoded G84R, G90S and Y111H mutations (presence of at least one versus absence of mutation in exon 3). To assess a putative effect of the genetic variants in an insulin-resistant subgroup the population was stratified according to the threshold of 27 for BMI. Association of the risk haplotype G-G with T2D was significant in the overweight group (P = 0.008), but not in the lean group (P = 0.19). However, the frequencies of the risk haplotype were similar in T2D patients from both groups (0.29), although the lean and overweight groups differed according to the frequencies of the risk haplotype G-G in normoglycemic subjects (0.26 and 0.24 respectively).

Sib-TDT analysis did not show transmission disequilibrium between the SNPs −11391 and −11377 risk haplotype and T2D. Indeed, the risk haplotype was equally transmitted to affected (n = 20) and unaffected (n = 21) individuals. Although the frequency of the risk haplotype G-G in T2D patients from this sample was similar (0.29) to that observed in the population, there was no association between the risk haplotype and T2D in our families (P = 0.9). No significant association was detected between insulin resistance (assessed by fasting insulinemia, the ratio of fasting insulinemia to fasting glycemia, and the HomaS index) and the risk haplotype G-G in normoglycemic subjects (0.26 and 0.24 respectively).

In Table 3, the haplotypes for the most relevant SNPs of the APMI gene are listed.

### Table 3. Haplotypes for the most relevant SNPs of the APMI gene

<table>
<thead>
<tr>
<th></th>
<th>−11426A &gt; G</th>
<th>−11391G &gt; A</th>
<th>−11377C &gt; G</th>
<th>−4041A &gt; C</th>
<th>−3971A &gt; G</th>
<th>+45T &gt; G</th>
<th>+276G &gt; T</th>
<th>+2019delA</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>l(T)</td>
<td>l(G)</td>
<td>l(C)</td>
<td>l(T)</td>
<td>l(G)</td>
<td>l(G)</td>
<td>l(G)</td>
<td>l(G)</td>
<td>l(G)</td>
<td>l(G)</td>
</tr>
<tr>
<td>l(A)</td>
<td>l(G)</td>
<td>l(C)</td>
<td>l(A)</td>
<td>l(A)</td>
<td>l(T)</td>
<td>l(T)</td>
<td>l(T)</td>
<td>l(T)</td>
<td>l(T)</td>
</tr>
<tr>
<td>l(G)</td>
<td>l(C)</td>
<td>l(C)</td>
<td>l(A)</td>
<td>l(A)</td>
<td>l(T)</td>
<td>l(T)</td>
<td>l(T)</td>
<td>l(T)</td>
<td>l(T)</td>
</tr>
</tbody>
</table>

1, wild allele; 2, variant allele. The corresponding nucleotides are shown as subscripts in parentheses.
DISCUSSION

In the present study, we have identified six new polymorphisms (SNPs $-11156$ insCA and $-11043$ C $>$ T and four rare mutations in exon 3) in French Caucasians, compared with previous data obtained in the Japanese population (15,17). Among the 10 common SNPs that we have extensively genotyped in the two populations, only SNP $-11377$ did not differ in allele and genotype frequencies between Caucasian and Japanese populations, indicating that both populations are significantly different regarding the $APM1$ gene’s SNP distribution. These differences may explain some discrepancies in linkage disequilibrium between T2D or adiponectin values and certain SNPs in the two ethnic groups—although, taken together, our present data confirm and amplify those previously found in Japanese diabetics, suggesting a contribution of the $APM1$ gene to the genetic risk for T2D (17).

Comuzzie et al. (18) recently showed a strong heritability for plasma adiponectin levels. Our results confirm a high heritability in French Caucasians, and indicate that plasma adiponectin levels are modulated by several variants of $APM1$, including SNPs $-11391$ and $-11377$ located in the $APM1$ proximal promoter, together with the rare non-synonymous mutations G90S and Y111H, both located in exon 3. Furthermore, haplotype analyses of the two promoter SNPs revealed a risk haplotype (G-G) associated with low plasma adiponectin levels and also with T2D ($P < 0.0001$ and $P = 0.004$, respectively). These results suggest a causal effect of impaired adiponectin secretion (genetically induced or due to other factors such as age and adiposity) in the development of T2D, confirming functional studies in animals.

Recently, an association of obesity and insulin sensitivity with the T $>$ G +45 polymorphism in exon 2 was reported in German subjects without familial history of T2D (19). We did not detect such an effect in the present study, which is not surprising, since most of our subjects belong to families with a strong familial history of T2D and/or obesity. A weak association between plasma adiponectin levels and SNPs +45 and +276 was noticed in our population, but the effect of these SNPs disappeared when we analyzed subjects with the CC genotype at SNP $-11377$ of the promoter, suggesting that their functional effect is at best marginal compared with the promoter SNPs. As stated earlier, some differences in genotype distribution between the French and Japanese populations may explain why our results differ from those reported by Hara et al. (17).

Adiponectin is known to modulate insulin resistance in mice (20). Furthermore, adiponectin concentration is correlated with insulin sensitivity in humans (11,21). However, we failed to detect in our French population a significant association between $APM1$ SNPs and insulin resistance indexes. These data contrast with previous reports (17,19). Several possible hypotheses may explain these discrepancies. First, the lack of robustness of insulin sensitivity indexes compared with euglycemic clamps, especially in diabetic subjects, is well known (22). In addition, it has been proposed that T2D in Japanese is rather a primary pancreatic disease (23,24), although in Caucasians insulin resistance is a major risk factor for diabetes, probably determined by several factors besides adiponectin levels. In some respects, adiponectin levels may be a better reflect of insulin resistance than indirect indexes are (21).

Alternatively, it is also possible that, in the French population, the SNP haplotypes most closely associated with adiponectin levels are predictive of a risk for T2D, independently of peripheral insulin sensitivity evaluated by HomaS. In this respect, it was recently reported that ACRP30 adiponectin administration has a striking effect on hepatic glucose output (10,25). Furthermore a direct positive effect of ACRP30 (and/or its globular head) on insulin secretion should not be neglected. Altogether, a decrease in adiponectin effect may have multiple tissue effects apart from the well-established increase in fatty acid oxidation in muscle, thus indirectly increasing glucose uptake and protecting from chronic hyperglycemia.

Taken together with previously published data, our study supports the idea that functional polymorphisms in $APM1$, modulating the level of fat-secreted adiponectin hormone, are associated with adiponectin levels and contribute to the genetic risk for T2D. Previous characterization of the human $APM1$ promoter did not report putative binding sites for transcription factors at and in the vicinity of SNPs $-11391$ and $-11377$ (26). However, computational analysis of a 91 bp sequence (nucleotides $-11435$/$-11345$) showed between SNPs $-11391$ and $-11377$, and adjacent to the $-11377$ position, a [tctgc] nucleotide sequence with similarity to the sequence of an enhancer element identified in the epidermal growth factor receptor (EGFR) gene and found in multiple sites protected by proteins (27). The association of the risk haplotype (G-G) and low plasma adiponectin levels could be partly explained by the presence of a polymorphic regulatory element.

The rare mutations reported here are not close to donor or acceptor splice sites. However, we cannot exclude the alteration of putative exonic splicing enhancers or silencers (28). The tertiary structure of adiponectin is rather complex, consisting of a head domain (until amino acid 45) followed by a collagen triple-helix region (until amino acid 110). The C-terminal part of adiponectin (globular domain), consisting of a bundle of collagen helices, is closely homologous to the complement protein C1q and displays structural homologies with proteins of the TNF family (29). The rare missense mutations detected here are located in the highly conserved collagen domain of the adiponectin protein from amino acids 45 to 110 (G84R and G90S), or at the hinge between the collagen and globular domains (Y111H). These residues are highly conserved between bovine, mouse and human (30). This region includes four lysine residues (at positions 68, 71, 80 and 104) that are also highly conserved between species, and were found to be hydroxylated and subsequently glycosylated together with proline 94, giving rise to six isoforms (31). The wild-type protein contains 22 Gly-X-Y or Gly-X-Pro repeats that form the collagenous triple helices. Mutations of glycine residues at positions 84 or 90 reduce the number of Gly-X-Y or Gly-X-Pro repeats to 14 and 16 respectively, thus disrupting the collagenous triple-helix structure. Mutation at position 111 near the hinge between the collagen and globular domains may partially hinder the complexation of collagenous homotrimeric in bundles. One can assume that these mutations may alter the spatial organization of the protein, either interfering with post-translational modifications involving lysine residues or modifying interactions of the protein with its receptor and...
underlying T2D. As the antibodies used to quantify adiponectin were directed against residues 32 to 244, the mutated protein could not be discriminated by the ELISA protocol if conformational changes modify accessibility to the antigenic part of the protein inducing the observed lower adiponectin levels.

Our data support the idea that functional polymorphisms in *APM1*, modulating the level of fat-secreted adiponectin hormone, are associated with adiponectin levels and contribute to the genetic risk for T2D, at least in French Caucasians. Nevertheless, the rare G90S and Y111H mutations of exon 3 are associated with the genetic risk of T2D [OR = 2.69 (IC = 1.14–6.36) and 2.48 (IC = 1.39–4.41), respectively]. Interestingly, the association with T2D remained significant (*P* = 0.003) when the rare mutations were included in the two promoter SNP haplotypes. Although the *APM1* gene is located in a region of linkage for early-onset T2D in our sample of families, we did not find association with the evidence of linkage, and family-based association analysis with T2D was not conclusive. This discordant result can be observed in several cases. First, the presence of several risk alleles can obscure the relationship between linkage and association. Second, it is possible that a main risk allele has not yet been discovered, but the effect of other variations of *APM1* is nonetheless detectable. Of course, we cannot rule out the possibility that alleles of this gene do not explain the observed linkage. Further family-based analyses are required to precisely define the role of the *APM1* haplotype in T2D.

In conclusion, from our study of 1373 subjects and 148 T2D nuclear families, common genetic variants defining a risk haplotype in the promoter and rare mutations in exon 3 are associated with serum adiponectin levels, and may be part of the genetic determinants for the risk of T2D in the French Caucasian population. From our results, Pritchard's question (32) asking if ‘rare variants (are) responsible for susceptibility to complex diseases?’, and challenging its counterpart the ‘common disease–common variant’ hypothesis (33), is not resolved. Indeed, the modulation of adiponectin levels and the potentially resulting increased susceptibility to T2D seem to be brought about by both common promoter variants and rare exonic mutations in the *APM1* gene. For example, the fraction of attributable risk of a rare mutation in exon 3 is estimated at 0.07. In this case, ignoring rare alleles when determining the genetic architecture of complex traits can be detrimental. Therefore, geneticists who dissect complex diseases should focus on both rare and common variants.

### MATERIALS AND METHODS

#### Subjects

1373 French Caucasians (mean age 57.6 ± 13.6 years, mean BMI 26.8 ± 7.5 kg/m² and sex ratio M/F = 604/769) and 743 subjects from 148 multiplex T2D families (12) were included in the study. Serum adiponectin levels were measured in 951 out of the 1373 subjects (mean age 58.1 ± 13.0 years, mean BMI 27.4 ± 6.7 kg/m² and sex ratio M/F = 401/550) and in subjects of the 148 multiplex families, as previously described (17). DNA was extracted from EDTA whole-blood samples using the Puregene kit (Gentra, Minneapolis, MN).

#### Screening of the *APM1* gene

We have screened for SNPs by direct sequencing in 40 unrelated type 2 diabetic French Caucasian subjects. The protocol was carried out using a 3700 DNA sequencer (Applied Biosystems, Foster City, CA) as previously described (34). Assigning positions according to the relative location to the A of the ATG (located in exon 2), we scanned 7.6 kb of the *APM1* gene, including (i) 874 bp from the 5' sequences, the exon 1 and part of intron 1 (positions −11499 to −10185) and (ii) part of intron 1, exon 2, intron 2, exon 3 and the 3' untranslated region 3'UTR (positions −4100 to +2200). Following the initial screening, as rare missense mutations were detected in exon 3 only, the screening of this exon was extended to 1246 out of the 1373 subjects, and in the 743 subjects from the 148 T2D families, by dHPLC and direct sequencing as previously described (35).

#### Genotyping SNPs in the *APM1* gene

Ten polymorphisms of the *APM1* gene were genotyped by direct sequencing or by the LightCycler technique (Roche, Manheim, Germany). The LightCycler assay is based on hybridization probes labelled with fluorescent dyes that allow fluorescence resonance energy transfer (36). SNP genotyping was carried out using melting curve analysis. Sequences of primers used for PCR direct sequencing and for the LightCycler assay are available from the authors.

#### Statistical analysis

Genotype frequencies in different groups were compared through chi-square or by likelihood tests combined with determination of *P*-value via permutations for allelic associations (37). Continuous variables were compared using the Wilcoxon–Kruskal–Wallis test. Haplotype frequencies and standardized linkage disequilibrium (D') were determined with the PM+EH+ software (38). Heritability was estimated in our sample of 148 families comprising sibships through a maximum-likelihood ratio test of the polygenic hypothesis. Calculation was performed with the QTDT program (39).

Adiponectin values were corrected for confounding variables (age, sex and BMI) according to the classical ordinary least-squares regression procedure. The equation for the present study is:

\[
\ln(\text{adiponectin}) = 0.917 + 0.005 \times \text{age} + 0.097 \times \text{sex} - 0.006 \times \text{BMI}.
\]

The orthogonal model, implemented in the same QTDT program, was used for family-based association test for quantitative trait (adiponectin, corrected for age, sex and BMI). This model separated allelic effect into orthogonal components, the between-family component being equivalent to the additive effect. This additive effect is given together with the *P*-value.
Finally, to explore the effect of haplotypes, we determined and assigned the most likely haplotype for each individual in families using the Genehunter software.

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